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DRUG DISCOVERY

Genetic and Molecular Mechanisms Associated with Antibiotic Resistance in Methicillin Resistant *Staphylococcus aureus* (MRSA): A Review

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Methicillin Resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen worldwide. It is still one of the major problems of drug resistance and it should be a frequent and an important human pathogen both in community and in hospital. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been known among the most important and intimidating bacteria involved in hospital infections in humans. MRSA resistance to methicillin has been attributed to a number of mechanisms, but the chief factor is reckon as its ability to produce specific binding protein 2a (PBP-2a) which renders β-lactamase resistant penicillins ineffective including all other β-lactam drugs. The Penicillin Binding Protein 2 has shown a usual low binding affinity for almost all beta-lactam antibiotics as compared to native PBPs. The Penicillin Binding Protein 2a (PBP2a) is coded and induced by the *mec A* gene a part of *Staphylococcal* cassette chromosome (*SCCmec*). The *SCCmec* is known to be present in MRSA but not in the MSSA strains. *SCCmec* is shown to be located in exactly the same region between *spa* and *purA* in the *S. aureus* chromosome. Another chromosomal gene called femA, working with *mecA* gene is required for the expression of MRSA and this gene is found to be absent in other Staphylococcus species giving *S. aureus* special feature to differentiate from other *Staphylococci*. Apart from *mec A* gene, a number of environmental and genetic factors have also shown to influence the methicillin resistance. The paper review the genetic and molecular mechanisms associated with beta-lactam antibiotics in MRSA.

INTRODUCTION

Staphylococcus aureus is a bacterium of significant importance because of its ability to cause a wide range of diseases and capacity to adapt to diverse environmental forms [1]. The organism colonizes skin, skin glands and mucous membrane, causing infections both in human and animals such as rashes, inflammations of bones and the meninges as well as septicaemia [2]. In addition, *S. aureus* causes inflammation of the mammary gland in bovine and the lower part of the foot in poultry [3]. *Staphylococcus aureus* remains one of the most significant pathogens causing disease in animals and human and methicillin-resistant *S. aureus* (MRSA) is ranked among the most important and common pathogens resistant to multiple antibiotics all over the world. Penicillin and its derivatives, including methicillin have been used for the treatments of infections caused by *S. aureus* [4]. However, certain strains of *S. aureus* developed resistance known as methicillin resistant *Staphylococcus aureus* (MRSA). At present, less than 90% of *S. aureus* strains are resistant to most penicillin derivatives [5] and ordinary antimicrobial agents like drugs from the family of aminoglycosides, macrolides, chloramphenicols, tetracyclines and fluoroquinolones [6]. A gene known as *mec*A gene is responsible for the resistance to methicillin which codes for penicillin- binding protein PBP 2A [7]. Lately, a new methicillin resistance mechanism gene, *mec*C was described in *S. aureus* [8]. García-Álvarez *et al*. [9], Paterson *et al*. [10], Wesse *et al*. [11] and Paterson *et al*. [12] reported MRSA isolates carrying *mec*C gene from humans and animals. Harrison *et al*. [13] suggested the public health hazard of *mecC*-positive MRSA isolates as it has been isolated in human case and their livestock. Until recently, MRSA was associated with prior exposure to health care facility, and as such, was considered a nosocomial pathogen [14].

METHICILLIN RESISTANCE *STAPHYLOCOCCUS AUREUS* (MRSA)

MRSA stands for Methicillin-Resistant *Staphylococcus Aureus*. The MRSA bacteria belong to the *Staphylococcus aureus* (*SA*) bacteria family. *Staphylococcus aureus* is a common bacterium. It lives harmlessly on the skin and in the nose of around a third of healthy people. When it does cause infection 'ordinary' *Staphylococcus aureus* is sensitive to most commonly used antibiotics. MRSA is a particular type of *SA* that has developed resistance to several antibiotics. Only a few antibiotics will kill MRSA [15].

The wide spread use of antibiotic resulted in the development of resistance to antibiotics through acquisition of the mobile cassette chromosome carrying the methicillin-resistant gene mecA [7] and mecC [8]. Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Methicillin-Resistant Coagulase-Negative *Staphylococci* (MR-CoNS)

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has been identified as multidrug-resistant zoonotic pathogens in humans and many animal species [16, 17]. Even through, coagulase negative *Staphylococci* may also be a normal flora for skin and mucous membranes of human and animal species [2].

The MRSA was first noted in 1961, about two years after the antibiotic methicillin was initially used to treat *Staph aureus* and other infectious bacteria. The resistance to methicillin was due to a penicillin-binding protein coded for by a mobile genetic element termed the methicillin-resistance gene –mecA [18]. In recent years, the gene has continued to evolve so that many MRSA strains are currently resistant to several different antibiotics such as penicillin, oxacillin and amoxicillin [19]. *Staphylococcus aureus* is one of the most important pathogens that can cause suppuration, abscess formation, a variety of pyogenic infection and even fatal septicemia in human beings. MRSA is still considered as an emerging pathogen and public health threats result from the spread of hospital-acquired as well as community-acquired MRSA [20]. The heterogeneous expression of methicillin resistance can make it difficult to determine the resistance phenotype definitively [21], therefore detection of the *mec*A gene remains the "gold standard" [22].

During the last decade, many studies have demonstrated the extremely high capacity of PCR for specifically detecting bacteria and genes of interest [23]. Several authors have already shown the feasibility of the PCR methodology for the identification of *S. aureus* strains and for the detection of antibiotic resistance genes [24]. MRSA is resistant to not only methicillin and other β-lactams but also may other antibacterial agents; therefore new agents are needed to treat the MRSA. The treatment of infectious diseases with antimicrobial agents continues to present problems in modern-day-medicine with many studies showing a significant increase in the incidence of bacterial resistance to several antibiotics [25]. Many plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit growth of pathogenic bacteria. Though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has developed. Medicinal plants are natural resources, yielding valuable products which are often used in the treatment of various ailments. Plant materials remain an important resource for combating illnesses, including infectious diseases, and many of the plants have been investigated for novel drugs or templates for the development of new therapeutic agents [26]. Most previous studies on plants for antibacterial activity were mainly performed with the extract of aerial parts of leaves, stem, flowers and ground level roots and rhizomes but meager research was done with seed extracts.

MRSA is a common pathogen responsible for skin and soft tissue infections, severe bloodstream infections, and pneumonia. MRSA was once a predominantly hospital acquired infection but in recent years has been increasingly found in community-onset infections. The proportion of *S. aureus* that is resistant to methicillin has declined in Europe and the United States over the past eight years, from 22 to 18 percent and from 53 to 44 percent, respectively, though the decrease has been slowing in Europe [27, 28, 29]. MRSA rates have also declined in Canada, from 21 to 16 percent since 2009, particularly in hospitals, but remain higher than pre-2000 rates [28,29]. In Australia, MRSA prevalence increased from 12 percent in 2000 to 19 percent in 2013 [28, 30]. In sub-Saharan Africa, MRSA prevalence increased in the early 2000s but has decreased since 2011 in South Africa (from 34 to 28 percent) [28, 31]. It also has begun to decline in South Africa (to 28 percent), where antibiotic stewardship is taking hold [28, 31]. However, in sub-Saharan Africa, India, Latin America, and Australia, it is still rising [28, 31], recorded at 90 percent in Latin American hospitals in 2013. But in India, a steep increase in MRSA, from 29 percent of *S. aureus* isolates in 2009 to 47 percent in 2014 [28], was recorded by a large private laboratory network. MRSA prevalence decreased in Thailand from 28 percent in 2009 to 19 percent in 2013 [32]. In 2013, MRSA accounted for 90 percent of all hospital *S. aureus* isolates from all but three countries in Latin America reporting to the Pan American Health Organization, ranging from zero in the Dominican Republic to 100 percent in Chile. In community settings, MRSA accounted for more than 80 percent of *S. aureus* isolates in all reporting countries except Bolivia. The proportion of MRSA ranged from 47 percent in Bolivia to 100 percent in Chile and the Dominican Republic [28].

HISTORICAL BACKGROUND OF MRSA

Alexander Fleming conducted a research and reported the bactericidal effects of a fungal contaminant that produced penicillin against *S. aureus* growing on culture plates [33]. A mass production of the drug from vats of cornsteep liquid growing on the mold was preceded due to the high mortalities during World War II [34]. Subsequently, there was a dramatic drop in death rates from bacterial pneumonia and meningitis in World War II compared to World War I. This led to the development of penicillin as the first major driver in selecting for resistant *S. aureus*. In 1940, an active β- lactam ring enzyme was described in *Escherichia coli* that are capable of hydrolyzing the penicillin. This enzyme was later named "penicillinase" [35] while in 1944; penicillinase production was also discovered in *S. aureus* [36]. In 1948, it was observed that over 50% of staphylococcal isolates recovered from patients in a United Kingdom hospital were resistant to penicillin [37]. Since then to date, 90 to 95% of *S. aureus* strains worldwide are penicillin resistant, with the plasmid encoded penicillinase readily transferable via transduction or conjugation. A penicillinase-resistant penicillin known as methicillin was introduced in 1959 to combat penicillin-resistant *S. aureus*, but within a year, late Professor Patricia Jevons reported the first human *S. aureus* strain to be methicillin resistant in UK hospital [38]. In 1962, an epidemic occurred at a hospital called Queen Mary's Children's Hospital, Carshalton. These strains became widespread in hospitals and into communities by the 1960's [39]. In 1968, United States recorded the first outbreak of MRSA [40] while in the 1970s, *S. aureus* strains have become resistant to most penicillinase-stable penicillins. It was first assumed to be a disease of human origin until when MRSA was first isolated in 1972 in a mastitic cow [41]. Thereafter, reports of MRSA infection became established in domestic and wild animals [41, 42].

EPIDEMIOLOGY OF MRSA

Epidemiological typing of MRSA strains resulted in the recognition of different lineages that are zoonotic, humanosis and/or host specific. Seventeen epidemic strains of human MRSA have been described in the United Kingdom [44] but the most dominant are EMRSA-15 and EMRSA-16 [45]. The EMRSA-16 clone represents major cause of human MRSA infections in Europe and America [46]. In Africa, epidemiological data on the predominant clones responsible for most epidemics is poorly documented. According to Breurec *et al* [47], the most predominant clones of African origin are ST88-IV, ST5-IV and ST239-III which are CA-MRSA. ST88-IV is a clone identified both in hospitals and community infections. The European lineage (EMRSA-16) has been described to originate from sub-saharan Africa [48] and has been reported in hospital and community acquired infections in Algeria [49]. Other lineages of human origin include CC1, CC5, CC8, CC22,

CC30 and CC45 while MRSA lineage predominant in pigs and other food animals is CC398 [50, 51]. Interspecies transmission of the strain CC398 (ST398) is a potential hazard and can be facilitated by frequent contact, environmental contamination and individual's immunity [52]. Three major settings were recognized according to host specification, reservoir and source of transmission [53].

MECHANISM OF RESISTANCE IN MRSA

β-lactam antimicrobial drugs target and inhibit bacterial cell-wall biosynthesis. Peptidoglycan is the main structural component of the cell-wall, and consists of glycan strands made of repeating *N*-acetylglucosamine and *N*-acetylmuramic acid disaccharides linked by peptide cross-links between *N*-acetylmuramic acid moieties on adjacent strands. Many of the steps in cell wall biosynthesis are similar between diverse bacteria and have been reviewed elsewhere [54], including specifically in staphylococci [55]. In brief, *N*-acetylglucosamine and *N*-acetylmuramic acid disaccharides are attached via a β 1,4-glycosidic bond to the reducing end of the growing peptidoglycan chain in a transglycosylation reaction. The newly incorporated repeating unit is cross-linked by a transpeptidation reaction to a stem peptide in an adjacent peptidoglycan strand. Transglycosylation and transpeptidation are both carried out by penicillin-binding proteins (PBPs), with the latter reaction being the specific target of β-lactams. Transpeptidation is a two-step reaction beginning with active-site serine-mediated acylation of the position 4 D-alanine carbonyl in the stem peptide. Deacylation of the resultant intermediate follows via nucleophilic attack of a side chain in the amino group in the third position of the stem peptide on an adjacent peptidoglycan strand. The stem peptide composition varies between bacterial species, but is typically comprised of L-alanine-γ-D-glutamate-diaminopimelate-D-alanine-D-alanine in Gram-negative bacteria, and L-alanine-γ-D-glutamate-l-lysine-D-alanine-D-alanine in Gram-positive bacteria such as staphylococci. In the case of *S. aureus*, cross-linking occurs between the position 4 D-alanine on one peptidoglycan strand and a pentaglycine extension attached to the position 3 L-lysine of the peptide stem of another [55]. Unlike the resistance of *S. aureus* to penicillin, methicillin resistance is not mediated by a plasmid-borne β-lactamase [56], and was referred to as intrinsic resistance in some of the early literature [57]. Similar intrinsic resistance to β-lactams in other bacterial pathogens had been shown to be associated with alterations in PBPs, either in their amount or in their affinity for β-lactams, and so attention turned to PBPs in MRSA as the likely mechanism. Alterations in PBPs specific to MRSA were noted but it was unclear if these related to over-expression and/or modification of existing PBPs, or the presence of a new PBP [57]. Hartman and Tomasz resolved the major difference between isogenic methicillin-resistant and susceptible strains by demonstrating the presence of a new PBP termed PBP2a with reduced affinity for β-lactams [58]. It is also designated as MRSA PBP in the early literature [59]. In contrast to β-lactamase, the gene(s) responsible for methicillin resistance (initially referred to as *mec*r for methicillin resistance) were located on the chromosome [60], which was traced to a region of 'foreign DNA' present in resistant but absent in susceptible strains [61]. Cloning and expression of the gene responsible in *E. coli* resulted in the heterologous expression of PBP2a [62]. This event was subsequently replicated in a *S. aureus* background [63] and the gene (later designated *mecA* [64] sequenced and shown to encode a PBP by Song *et al*. [59]. Confirmation of the role of *mecA* in conferring methicillin resistance came from the transfer and β-lactams inhibit the transpeptidation step of cell-wall biosynthesis by acting as substrate analogs of the D-ala-D-ala peptidoglycan side chain upon which PBPs act [65]. A long-lived covalent acyl-enzyme complex forms between the β-lactam and the nucleophilic serine of the PBP active site, which inhibits cell-wall transpeptidation. Deacylation of this complex as occurs during normal turnover is impeded because the region of the active-site that accommodates the deacylating acceptor moiety or a potential hydrolysing water molecule is occupied by the β-lactam ring structure. Regeneration of the PBP is so slow relative to cell division that the enzyme is effectively irreversibly inactivated. The consequential loss of cell wall cross-linking leads to defective cross-wall formation during cell division, and cell death. The exact mechanisms leading to this cell death are poorly understood. A role in some bacterial species is observed for autolytic enzymes causing cell-wall degradation and lysis [65]. However, mechanistic understanding is complicated by variable responses to the same β-lactam by different bacterial species, and variable responses within the same bacterial species to different β-lactams [65]. In the case of staphylococci, there is evidence that the high internal pressure causes cytoplasm leakage through the weakened peripheral wall leading to death [55].

Resistance conferred by PBP2a manifests through both a reduced rate of β-lactam-mediated enzyme acylation ($k2$) compared to native PBPs, and an absence of high affinity for β-lactam in the first instance (Kd). The second-order rate constant $k2/Kd$ is taken as a measure of the inhibitory potential of β-lactams against PBP. For PBP2a this rate constant varies between different β-lactams and experimental approaches [66] and is 2--3 orders of magnitude lower than that seen for β-lactam-susceptible PBPs, including native PBP2 from *S. aureus* [67]. Once acylated, PBP2a undergoes deacylation at a slow rate, comparable to β-lactam-susceptible PBPs with a half-life value for the acyl-enzyme of up to 77 hours [66]. Crystal structures of PBP2a from MRSA have been resolved, providing the structural basis for resistance. Overall, PBP2a forms an elongated protein with a transpeptidase domain (residues 327--668) and what was referred to in the first crystal structure report as a nonpenicillin-binding domain (residues 27-138) [68] and subsequently shown to contain an allosteric site [69]. The full-length protein also possesses a transmembrane domain (residues 1--23), which is typically removed to produce a soluble protein amenable to study. Significantly with regards to resistance, the active-site serine of PBP2a is less accessible to β-lactams than susceptible PBPs because of its location in a narrow extended cleft. The inaccessibility of the active site mean that the slow deacylation of the inactivated acyl-enzyme intermediate displayed by PBP2a, and shared with β-lactam-susceptible PBPs, does not affect the clinical resistance of MRSA as this intermediate does not form at the concentrations of antibiotic reached *in vivo*. Furthermore, acylation appears to require a conformational change that makes this reaction less favourable than in susceptible enzymes [68]. Another significant structural and functional feature of PBP2a, unlike other PBPs, is that it is under allosteric control [69].

Allostery had first been predicted from kinetic studies showing an increased rate of PBP2a acylation in the presence of synthetic fragments of peptidoglycan [66]. Allosteric control of PBP2a was subsequently confirmed with structural analysis and the allosteric site identified within the nonpenicillin-binding domain [69]. Binding of nascent peptidoglycan at this allosteric site, 60 Å removed from the active-site, stimulates conformational changes through a series of salt-bridge interactions which result in opening of the active-site to facilitate substrate access [69]. Binding of peptidoglycan at the allosteric site appears to be through recognition of the D-Ala-D-Ala terminus of the pentapeptide stem and thus functions to open the active-site for

transpeptidation only in the presence of the nascent peptidoglycan substrate [69].

SCC*mec* Sequencing of the region containing *mecA* revealed a distinct mobile genetic element named the staphylococcal chromosome cassette (SCC*mec*) that was present in MRSA but absent in methicillin-susceptible *S. aureus* (MSSA) [70]. SCC*mec* elements are highly diverse and eleven types (I to XI) have been recognised to date [71]. Despite their diversity in size (from approximately 21 kb to 67 kb) and gene content, they all share important defining characteristics. In all cases, SCC*mec* is integrated into the *S. aureus* genome at an *attB* integration site sequence present at the 3' end of the *orfX* gene. Despite its significance as the site of SCC*mec* integration, the function of *orfX* was unresolved for many years until analysis of its crystal structure revealed structural homology to ribosomal methyltransferase of the RlmH type [72]. Insertion of SCC*mec* into *orfX* does not alter *orfX* expression because the terminal amino acids and stop codon at the insertion site are unchanged, even though the DNA sequence is slightly altered [72]. Another shared feature is that all SCC*mec* elements contain a *mec* gene complex comprising *mecA* and its regulatory genes *mecI* and *mecR* (although *mecI* and *mecR* are not intact in some SCC*mec* classes), a cassette chromosome recombinase (*ccr*) gene complex containing one or two site-specific recombinase genes responsible for movement of the SCC*mec*, and typically three J regions. Originally designated junkyard regions due to the presence of pseudogenes and truncated copies of transposons and insertion sequences, these J regions are now commonly referred to as joining regions in the recognition that they can encode important functions such as resistance to additional antibiotics and resistance to heavy metals. The other shared feature of SCC*mec* elements is their demarcation by specific inverted repeats and direct repeats containing the insertion site sequence recognised by the *ccr*-encoded recombinases [72].

SCC*mec* typing is widely used for epidemiological surveillance of MRSA and classifies SCC*mec* elements based on their combination of *mec* gene and *ccr* gene complexes, with further subtyping based on the J regions [73]. Two distinct *ccr* complexes have been described to date, the first comprising *ccrA* and *ccrB*, and the second consisting of a single *ccrC* gene. Sequence variation among *ccrA* and *ccrB* defines several allotypes (where nucleotide identity of <50% defines a new gene and novel allotypes of *ccr* genes designated if their DNA sequence identities are between 50% and 84%, with an allele sharing 85% nucleotide identity). The combination of *ccrA* and *ccrB* defines the *ccr* gene complex type, designated as type 1 (*ccrA1B1*), type 2 (*ccrA2B2*), type 3 (*ccrA3B3*), type 4 (*ccrA4B4*), type 7 (*ccrA1B6*) and type 8 (*ccrA1B3*). In contrast, reported *ccrC* variants show a high degree of similarity and only one allotype *ccrC1* has been defined so far which constitutes *ccr* gene complex type 5. In addition to *mecA* and its regulatory genes, the *mec* gene complex includes associated insertion sequences, with five *mec* gene complex types currently recognized largely based on the presence and location of these insertion sequences. An International Working Group on the Classification of Staphylococcal Cassette Chromosome (SCC) Elements (IWG-SCC) was established to provide consensus guidelines on this nomenclature [73]. A variety of PCR-based protocols are currently used for SCC*mec* typing, which are likely to become superseded by data from whole genome sequencing [73].

CONCLUSION

Staphylococcus aureus is a major human and veterinary pathogen worldwide. Methicillin-resistant *S. aureus* (MRSA) poses a significant and enduring problem to the treatment of infection by such strains. Resistance is usually conferred by the acquisition of a non-native gene encoding a penicillin binding-protein (PBP2a), with significantly lower affinity for β-lactams. This allows cell-wall biosynthesis, the target of β-lactams, to continue even in the presence of typically inhibitory concentrations of antibiotic. PBP2a is encoded by *mecA* gene carried on a distinct mobile genetic element, SCC*mec*, the expression of which is controlled through a proteolytic signal transduction pathway comprising a sensor protein MecR1 and a repressor MecI. Many of the molecular and biochemical mechanisms underlying methicillin resistance in *S. aureus* have been elucidated, including regulatory events and the structure of key proteins.

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